PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/46, 7/08, A61K 35/56 — A1

(11) International Publication Number: WO 98/25961

(43) International Publication Date: 18 June 1998 (18.06.98)

(21) International Application Number: PCT/SE97/02075

(22) International Filing Date: 12 December 1997 (12.12.97)

(30) Priority Data:
9604593-5 13 December 1996 (13.12.96) SE

(71) Applicant *(for all designated States except US)*: SBL VACCIN AB [SE/SE]; S-105 21 Stockholm (SE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BARRA, Donnatella [IT/IT]; University of Rome, Dept. of Biochemical Sciences, La Sapienza Piazzale Aldo Moro, 5, I-00185 Rome (IT). SIMMACO, Maurizio [IT/IT]; University of Rome, Dept. of Biochemical Sciences, La Sapienza Piazzale Aldo Moro, 5, I-00185 Rome (IT).

(74) Agent: AWAPATENT AB; P.O. Box 45086, S-104 30 Stockholm (SE).

(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANTIMICROBIALLY ACTIVE POLYPEPTIDES

F L P L I G R V L S G I L - amide
L L P I V G N L L K S L L - amide
L L P I L G N L L N G L L - amide
L L P I V G N L L N S L L - amide
V L P I I G N L L N S L L - amide
F L P L I G K V L S G I L - amide
F F P V I G R I L N G I L - amide
L S P N L L K S L L - amide
L L P N L L K S L L - amide
F V Q W F S K F L G R I L - amide

(I)

G L L S G L K K V G K H V A K N V A V S L M D S L K C K I S G D C

(57) Abstract

A polypeptide selected from peptides (I) and functional derivatives and pharmaceutically acceptable salts thereof; pharmaceutical compositions containing one or more of these polypeptides; and a method for inhibiting microbial growth in animals using such polypeptides.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

ANTIMICROBIALLY ACTIVE POLYPEPTIDES

The present invention relates to new polypeptides for therapeutic use and their functional derivatives and pharmaceutically acceptable salts. The new polypeptides have each per se or in combination of one or more of the peptides antibacterial or fungal use.

Skin secretions of frogs contain many different types of antibacterial peptides (Barra, D. & Simmaco, M. (1995) Amphibian skin: a promising resource for antimicrobial peptides, Trends Biotechnol. 13, 205-209 for a recent review). In particular, a variety of such peptides has been isolated from several *Rana* species. They all contain two cysteine residues close to the COOH-terminus which form an intramolecular disulfide bridge. Four different groups of these peptides can be discerned. One is the brevinin 1 family, which includes brevinin 1 from *Rana brevipoda porsa* (Morikawa, N., Hagiwara, K. & Nakajima, T. (1992) Brevinin-1 and Brevinin-2, unique antimicrobial peptides from the skin of the frog, *Rana brevipoda porsa*, Biochem. Biophys. Res. Commun. 189, 184-190), brevinin 1E from *Rana esculenta* (Simmaco, M., Mignogna, G., Barra, D. & Bossa, F. (1994) Antimicrobial peptides from skin secretion of *Rana esculenta*. Molecular cloning of cDNA encoding esculentin and isolation of new active peptides, J.Biol.Chem. 269, 11956-11961), ranalexin from *Rana catesbeiana* (Clark, D.P., Durell, S., Maloy, W.L. & Zasloff, M. (1994) Ranalexin, a novel antimicrobial peptide from bullfrog (*Rana catesbeiana*) skin, structurally related to the bacterial antibiotic, polymixin, J.Biol. Chem. 269, 10849-10855) and gaegurin 5 and 6 from *Rana rugosa* (Park, J.M., Jung, J.-E. & Lee, B.J. (1994) Antimicrobial peptides from the skin of a korean frog, *Rana rugosa*, Biochem. Biophys. Res. Commun. 205, 948-954). These peptides are composed of 20-24 amino acid residues. In addition to their antibacterial action, brevinin 1E and ranalexin also have high hemolytic activity. A second group are the brevinin 2 peptides, which contain 29-34 amino acids. Besides brevinin 2 *from R. brevipoda porsa* (Morikawa,

N., Hagiwara, K. & Nakajima, T. (1992) Brevinin-1 and Brevinin-2, unique antimicrobial peptides from the skin of the frog, *Rana brevipoda porsa*, Biochem. Biophys. Res. Commun. 189, 184-190), several peptides from *R. esculenta* (Simmaco, M., Mignogna, G., Barra, D. & Bossa, F. (1994) Antimicrobial peptides from skin secretion of *Rana esculenta*. Molecular cloning of cDNA encoding esculentin and isolation of new active peptides, J.Biol.Chem. 269, 11956-11961), the gaegurins 1-3 (Park, J.M., Jung, J.-E. & Lee, B.J. (1994) Antimicrobial peptides from the skin of a korean frog, *Rana rugosa*, Biochem. Biophys. Res. Commun. 205, 948-954) and rugosins A and B from *R. rugosa* (Suzuki, S., Ohe, Y., Okubo, T., Kakegawa, T. & Tatemoto, K. (1995) Isolation and characterization of novel antimicrobial peptides, rugosin A, B and C, from the skin of the frog, *Rana rugosa*, Biochem. Biophys. Res. Commun. 212, 249-254) belong to this family. A third group are the 37 residue peptides esculentin 2 from *R. esculenta* (Simmaco, M., Mignogna, G., Barra, D. & Bossa, F. (1994) Antimicrobial peptides from skin secretion of *Rana esculenta*. Molecular cloning of cDNA encoding esculentin and isolation of new active peptides, J.Biol.Chem. 269, 11956-11961) and gaegurin 4 (Park, J.M., Jung, J.-E. & Lee, B.J. (1994) Antimicrobial peptides from the skin of a korean frog, *Rana rugosa*, Biochem. Biophys. Res. Commun. 205, 948-954) and rugosin C from *R. rugosa* (Suzuki, S., Ohe, Y., Okubo, T., Kakegawa, T. & Tatemoto, K. (1995) Isolation and characterization of novel antimicrobial peptides, rugosin A, B and C, from the skin of the frog, *Rana rugosa*, Biochem. Biophys. Res. Commun. 212, 249-254). Lastly, esculentin 1 from skin secretion of *R. esculenta* (Simmaco, M., Mignogna, G., Barra, D. & Bossa, F. (1994) Antimicrobial peptides from skin secretion of *Rana esculenta*. Molecular cloning of cDNA encoding esculentin and isolation of new active peptides, J.Biol.Chem. 269, 11956-11961), a 46 amino acid peptide that has the highest antibacterial activity of all the *Rana* peptides characterized so far. In addition, it is also active against *Candida albicans*,

Saccharomyces cerevisiae and *Pseudomonas aeruginosa*.

The present invention has for an object to provide relatively small polypeptides of antimicrobial activity.

Another object of the invention is to provide such new polypeptides having antibacterial or fungal use.

Yet another object of the invention is to provide pharmaceutical compositions containing one or more such polypeptides contained in a pharmaceutically acceptable matrix.

Still another object of the invention is to provide a method for inhibiting microbial growth in animals, such as mammals including man.

For these and other objects which will be clear from the following disclosure the invention provides for the following new peptides:

F L P L I G R V L S G I L - amide
L L P I V G N L L K S L L - amide
L L P I L G N L L N G L L - amide
L L P I V G N L L N S L L - amide
V L P I I G N L L N S L L - amide
F L P L I G K V L S G I L - amide
F F P V I G R I L N G I L - amide
L S P N L L K S L L - amide
L L P N L L K S L L - amide
F V Q W F S K F L G R I L - amide
G L L S G L K K V G K H V A K N V A V S L M D S L K C K I S G D C

Particularly preferred polypeptides are the following:

F L P L I G R V L S G I L - amide
L L P I V G N L L K S L L - amide
F L P L I G K V L S G I L - amide
F F P V I G R I L N G I L - amide
F V Q W F S K F L G R I L - amide

Within the scope of the invention there are also inclu-

ded functional derivatives and pharmaceutically acceptable salts of the polypeptides mentioned above.

The polypeptides according to the present invention can be used each per se or can be used in combinations of two or more polypeptides.

The polypeptides are therapeutically useful, such as for antimicrobial use, including antibacterial or fungal use.

The invention also provides for the use of one or more of the polypeptides disclosed above for the manufacture of a medicament having antimicrobial activity.

Furthermore, the invention provides for a pharmaceutical composition containing as an active ingredient one or more polypeptides as described above in an effective amount together with a pharmaceutically acceptable carrier or diluent. Said carrier or diluent is suitably adapted for oral, intraveneous, intramuscular or subcutaneous administration.

According to the invention there is also provided a cDNA clone having the sequence selected from the sequences shown as clone Rt-5, Rt-6 and Rt-17 as disclosed in the following.

Finally, the invention provides for a method for inhibiting microbial growth in animals, such as mammals including man, comprising the step of administering to an animal subject to a disorder caused by antimicrobial attack one or more polypeptides as described above or a composition thereof, an inhibitory amount being administered.

Such method can be directed to intestinal use constituted by oral administration of a composition as defined above in a slow release form. The method can also be directed to administration by injection of such a composition in an injectable dose form.

With regard to the expression "functional derivatives thereof" it is well known in regard to the technical area to which the present invention pertains that minor amino acid substitutions can be made to the polypeptide which do not affect or do not substantially affect the function of the polypeptide. Determination of conceivable substitutions is ac-

complished according to procedures well known to those skilled in the art. Thus, all polypeptides having substantially the same amino acid sequence, substantially the same helical structure and substantially the same biological activity, such as antimicrobial and lytic activity, are within the scope of this invention.

Also within the scope of the present invention are pharmaceutically acceptable salts of the polypeptides of this invention. Such salts are formed by methods well known to skilled artisans. Thus, for example base salts of the polypeptides can be prepared according to conventional methods. When in the instant disclosure including the claims the term polypeptide is used said term is intended to include both functional derivatives and pharmaceutically acceptable salts of the polypeptides.

The active polypeptide according to the present invention can be formulated for use in human or veterinary medicine for therapeutic or prophylactic use. The active preparations are normally administered orally, rectally or parenterally, such as by injection in the form of a pharmaceutical preparation or composition comprising the active constituents in combination with a pharmaceutically acceptable carrier which may be solid, semi-solid or liquid, or contained in a capsule, such as when orally administered. The administration may also take the form of topical application. As examples of pharmaceutical preparations there may be mentioned tablets, drops, solutions and suppositories. Usually, the active constituent constitutes the minor part of the preparation, such as from about 0.1 to about 50% thereof based on weight.

In order to prepare pharmaceutical compositions in the form of dose units for oral application the polypeptide of the invention can be mixed with a solid, pulverulent or other carrier, for example lactose, saccharose, sorbitol, mannitol, starch, such as potatoe starch, corn starch, millopectine, cellulose derivative or gelatine, and may also include lubricants, such as magnesium or calcium stearate, or polyethylene

glycol waxes compressed to the formation of tablets or bodies for dragées. The dose units may also be presented in a coated form of enteric type.

By using several layers of the carrier or diluent tablets operating with slow release can be prepared.

Liquid preparations for oral application or for injection can be made in the form of elexirs, syrups or suspensions, for example solutions containing from 0.1 to 20% by weight of active substance, sugar and a mixture of ethanol, water, glycerol, propyleneglycol and possibly other additives of a conventional nature.

The dose by which the active constituent is administered may vary within wide limits and is dependent on different factors, such as the seriousness of the disorder, the age and the weight of the patient and can be adjusted individually.

In finding the new polypeptides according to the present invention the skin of *Rana temporaria*, a red frog found in many parts of Central Europe, was used. A cDNA library prepared from the skin of this frog was screened with a DNA fragment encoding the signal peptide of the precursor of esulentin 1 from *R. esculenta*. Using this approach several clones could be isolated with inserts that potentially coded for the precursors of new peptides. The new peptides which could be isolated from skin secretion of *R. temporaria* were termed temporins and were found to have biological activities, such as antibacterial activity, both each per se and in synergistic combinations.

The present invention will now be described by non-limiting examples through the following disclosure. This disclosure is made with reference to the appended drawings, wherein:

Fig. 1 shows the nucleotide sequences of 3 clones and inserts present therein and also deduced amino acid sequences; and

Fig. 2 shows a diagram on reverse-phase HPLC of skin secretion of *R. temporaria*.

MATERIALS AND METHODS

Enzymes and Reagents. Analytical grade chemicals were from Merck, HPLC-grade solvents from Carlo Erba, sequenal-grade chemicals from Perkin Elmer. Media for antimicrobial assays were from Difco, agarose (A6013) from Sigma. Restriction enzymes and DNA modifying enzymes were from Boehringer Mannheim, deoxyribonucleotides from Pharmacia. DNA sequences were determined with a "Sequenase kit" (version2.0, U.S. Biochemicals) using [a-^{35}S]dATP. Synthetic peptides were purchased from TANA laboratories (Huston, USA).

Isolation of RNA and cloning procedure. For these studies the skin of two specimens of R.temporaria was used. The isolation of poly(A)-rich RNA by affinity chromatography over oligo(dT)-cellulose and the preparation of the cDNA library were performed according to Richter et al., (1990b). (Richter, K., Egger, R. & Kreil, G. (1990b) Molecular cloning of a cDNA encoding the bombesin precursor in skin of *Bombina variegata*, FEBS Lett. 262, 353-355.)

A cDNA library comprising about 10,000 clones was screened with a 240 bp fragment obtained by digestion of the esculentin 1 cDNA with HindIII (Simmaco, M., Mignogna, G., Barra, D. & Bossa, F. (1994) Antimicrobial peptides from skin secretion of *Rana esculenta*. Molecular cloning of cDNA encoding esculentin and isolation of new active peptides. J.Biol.Chem. 269, 11956-11961). This fragments encodes the prepro-region of the esculentin 1 precursor. The probe was labelled by random priming (Boehringer Mannheim). Hybridization was performed at 55°C for 16 h in 100 mM sodium phosphate buffer, pH 7,2, containing 850 mM NaCl, 1 mM EDTA, 10x Denhardt´s solution, 0,1% SDS and 100 mg/ml yeast tRNA. Filter papers (Whatman 541, 11 cm x 11 cm) were washed twice for 15 min at 50°C with SSPE (0.3 M NaCl, 20 mM sodium phosphate, pH 7,4, 2 mM EDTA), 0.2% SDS. Positive clones were selected and analysed by cleavage with restriction enzymes and nucleotide sequencing.

Northern blot analysis. Poly(A)-rich RNA (5 mg) was fractionated by electrophoresis in 1.2% agarose gels containing 0.8 M formaldehyde (Arrand, J.E. (1985) Preparation of nucleic acid probes, in Nucleic acid hybridization: a practical approach (Hames, B.D. & Higgins, S.J., eds) pp 17-45, IRL Press, Oxford) and blotted directly onto Nytran sheets (Schleicher & Schuell). The insert of clone Rt-17 was labeled by random priming and used for probing the Northern blot. Filters were washed at 55°C in 0.1 x SSPE, 0.1% SDS, and then used for autoradiography.

Collection and purification of skin secretions. Three specimens of R.temporaria (30-35 g each) were captured near Salzburg (Austria). They were maintained in a terrarium in our laboratory for 1 year and feed larvae of Tenebrio molitor. Skin secretions were collected at intervals of three weeks by a mild electrical shock (12 V, feet to head). The secretion was collected from the surface of a single frog by washing its dorsal region with 10 ml 0.05% (by vol.) acetic acid. The secretions of the three frogs were combined and lyophilized. Suitable aliquots were fractionated by HPLC on a Beckman model 332 system using a reverse-phase column (Aquapore RP-300, 7 mm x 250 mm. Applied Biosystems) eluted with a gradient of 10-70% acetonitrile/isopropanol (4:1) in 0.2% (by vol.) trifluoroacetic acid, at a flow rate of 1.8 ml/min. Elution of the peptides was monitored on a Beckman 165 spectrophotometer at 220 nm. Peak fractions were collected and lyophilized. A small aliquot of each peak was subjected to N-terminal analysis following derivatization with dansyl chloride and reverse phase HPLC separation (Simmaco, M., De Biase, D., Barra, D. & Bossa, F. (1990b) Automated amino acid analysis and determination of amidated residues using pre-column derivatization with dansyl-chloride and reverse-phase high performance liquid chromatography, J. Chromatogr. 504, 129-138). Further purification of peptides was achieved using a macroporous C_{18} column (4.6 mm x 150 mm, Supelco) developed with an appropriately modified gradient of the same

solvent system as described above.

Structural analysis. Amino acid analyses were performed with a Beckman System Gold analyzer, equipped with an ion-exchange column and ninhydrin derivatization, after vapor phase hydrolysis of the peptides (1-2 nmol) in 6 N HCl for 24 h. Peptide sequences were determined by automated Edman degradation with a Perkin-Elmer model AB476A sequencer. In some cases, information on the amidation state of the C-terminus was confirmed by mass spectral analysis and/or carboxypeptidase Y digestion (Simmaco, M., De Biase, D., Barra, D. & Bossa, F. (1990b) Automated amino acid analysis and determination of amidated residues using pre-column derivatization with dansyl-chloride and reverse-phase high performance liquid chromatography, J. Chromatogr. 504, 129-138).

Antimicrobial assay. The antibacterial activity was tested against Bacillus megaterium BM11, Staphylococcus aureus Cowahl, Streptococcus pyogenes b hemolytic group A, Pseudomonas aeruginosa ATCC 15692, Escherichia coli D21, E.coli D21e7, E.coli D21f1, E.coli D21f2 and E.coli D22, using an inhibition zone assay on LB broth/1% agarose plates seeded with $2x10^5$ viable bacteria (Hultmark, D., Engström, Å., Andersson, K., Steiner, H., Bennich, H. & Boman, H.G. (1983) Insect immunity. Attacin, a family of antibacterial proteins from *Hyulophora cecropin*, EMBO J. 2, 571-576). Fresh cultures of Candida albicans ATCC 10261 were inoculated in WB broth, pH 6.5, and grown at 37°C to approximately 0.6 OD_{600}. Before plating, cultures were diluted 300 fold and then incubated overnight at 37°C in the presence of the test peptide, the concentration of which was established by amino acid analysis. Inhibition zones were measured and the lethal concentration (LC, the lowest concentration that inhibits the growth) was calculated from the diameter of the zones obtained in serial dilutions of the test substance by using the formula given in Hultmark, D., Engström, Å., Andersson, K., Steiner, H., Bennich, H. & Boman, H.G. (1983) Insect immunity. Attacin, a family of antibacterial proteins from *Hyulophora ce-*

cropin, EMBO J. 2, 571-576). Values are expressed as the mean of at least 5 experiments with a divergence of not more than one dilution step.

Circular dichroism measurements. CD measurements were carried out on a Jasco J710 spectropolarimeter, equipped with a DP 520 processor, at 20°C, using a quartz cell of 2 mm pathlength. CD spectra were the average of a series of 3 scans. Ellipticity is reported as the mean molar residue ellipticity (q), expressed in deg cm^2dmol^{-1}. Peptide concentrations were determined by amino acid analysis.

RESULTS

Analysis of cDNA clones encoding the precursors. A 240 bp HindIII fragment encoding the signal peptide and the propart of the esculentin 1 precursor was used as a probe to screen the cDNA library prepared from skin of R.temporaria. Six positive clones were detected. The sequences of the inserts present in clones Rt-5, Rt-6 and Rt-17 are shown in Fig. 1. Excluding the poly(A) tail, these cDNAs comprise 323, 356 and 329 nucleotides, respectively. After the first methionine codon, they contain open reading frames which can be translated into polypeptides containing 58 (Rt-6) or 61 amino acids (Rt-5 and Rt-17). The deduced sequences all have the typical features of peptide precursors. They start with a signal peptide containing a cluster of hydrophobic residues. The cleavage site for signal peptidase is most likely located after the cysteine residue at position 22 (von Heine, G. (1983) Patterns of amino acids near signal-sequence cleavage sites, Eur. J. Biochem. 133, 17-21). The sequences of the putative mature peptides are preceded by a Lys-Arg, a typical processing site for prohormone convertases. All these precursors polyptpeides terminate with the sequence Gly-Lys. Hydrolysis by carboxypeptidase E would expose a C-terminal glycine which is required for the formation of COOH-terminal amides. The predicted end products would be amidated peptides containing 13 amino acids for clones Rt-5 and Rt-17, while

Rt-6 has a 9 bp deletion in this region, thus codes only for a decapeptide.

Northern blot analysis. In poly(A)-rich RNA from skin of R.temporaria, the probe derived from clone Rt-17 recognized an abundant message, detected as a single, rather broad band in the range of 400-500 nucleotides. Under the same conditions, no signal could be obtained from the skin of other amphibian species such as R.esculenta, Xenopus laevis and Bufo viridis.

Isolation and analysis of peptides from skin secretion. After electrical stimulation of 3 specimens of R.temporaria, about 20 mg of lyophilized material could be obtained. After a preliminary HPLC purification (Fig. 2), each fraction was subjected to N-terminal analysis, in order to identify those with amino-terminal Leu or Phe as predicted from the cDNA sequences. The relevant fractions were further purified by HPLC and subjected to amino acid and sequence analysis. Following this approach, the three predicted peptides were found to be indeed present in the secretion. Other molecules, structurally related to these peptides, were also isolated. The sequences of these peptides, which are termed temporins, are shown in Table 1. In this Table the amount of each peptide recovered from the secretion is also included. Along the HPLC profile reported in Fig. 2, the elution position of the various peptides is indicated. The structure of temporin E, with Val at its N-terminus, and which coeluted in part with temporin D, is also shown in the Table. Temporins are all amidated at their C-terminus, as predicted from the structure of the precursors (see above), and contain a prevalence of hydrophobic amino acids. Each of these peptides contains 13 residues, with the exception of temporins H and K, which are 10 residue long. Except for temporins C, D, and E, all of these peptides have at least one basic residue (either Lys or Arg). In the course of this analysis, a 22-residue peptide was also found in the skin secretion (see Table 1). Its sequence shows some similarity with that of melittin, a hemo-

lytic peptide from bee venom (Habermann, E. (1972) Bee and wasp venoms, Science 251, 1481-1485). It was thus named melittin-like peptide (MLP).

Assays for biological activity. The antimicrobial activity of the purified temporins was first tested against B.megaterium and E.coli D21. Temporins A, B, F, G and L were active on both bacterial strains, whereas temporins C, D, E, H and K only showed some activity against B.megaterium, the most sensitive bacterium.

The recovery of some of the temporins was too low to allow a detailed characterization of their biological properties. To confirm the structure and in order to obtain more material temporins A, B, D and H were chemically synthesized. The antimicrobial activity of synthetic temporins A and B, expressed as lethal concentration values, is reported in Table 2, together with the results obtained on red blood cell lysis. As references are included esulentin 1 from *R. esculenta* (Simmaco, M., Mignogna, G., Barra, D. & Bossa, F. (1994) Antimicrobial peptides from skin secretion of *Rana esculenta*. Molecular cloning of cDNA encoding esculentin and isolation of new active peptides, J.Biol.Chem. 269, 11956-11961), cecropin from insect hemolymph (Steiner, H., Hultmark, D., Engström, Å, Bennich, H. & Boman, H.G. (1981) Sequence and specificity of two antibacterial proteins involved in insect immunity, Nature 292, 246-248) and melittin from honeybee venom (Habermann, E. (1972) Bee and wasp venoms, Science 251, 1481-1485).

Synthetic temporins A and B showed the same activities as their natural counterparts while temporins D and H were found to be without any biological activity of their own or as enhancers of other *Rana* peptides. Temporin A is about three times more active than temporin B against *S. aureus* and *S. pyrogenes*. On the other hand, these two temporins were poorly active against *E.coli* D21 and completely inactive against *P. aeruginosa*. This indicates that temporins A and B act specifically against gram-positive bacteria.

Linear sulfur free antibacterial peptides like cecropins are inactive against fungi while the defensins (with three S-S bridges) show antifungal activity. Temporins A and B are active against *C. albicans*. and their potency is of the same order as reported for dermaseptin from the South American frog *Phyllomedusa Sauvagei* (Mor, A., Hani, K. & Nicolas, P. (1994) The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms, J.Biol.Chem. 269, 31635-61641).

The antibacterial activity of temporins A and B was also tested against three strains of *E.coli* D21, D21e7, D21f1 and D21f2, with consecutive mutations deleting increasing amounts of the side chain in LPS (Boman, H.G. & Monner, D.A. (1975) Characterization of lipopolysaccharides from *Escherichia coli* K12 mutants, J. Bacteriol. 121, 455-464). Strain D22 has a permeable outer membrane due to a mutation in the envA gene (Normark, S., Boman, H.G. & Matsson E. (1969), Mutant of *Escherichia coli* with anomalous cell division and ability to decrease episomally and chromosomally mediated resistance to ampicillin and several antibiotics. J.Bacteriol. 97, 1334-1342). The activities of the temporins were tested in the absence or in the presence of the basal medium E (Vogel, H.J. & Bonner, D.M. (1956) Acetylornithinase of *Escherichia coli*: partial purification and some properties, J.Biol.Chem. 218, 97-106).

The results in Table 3 show medium E was found to increase the activity of tempors in all strains tested. However no similar effects were seen with gram positive bacteria. CD spectra showed that the increase in activity was correlated to an increased helix formation as found before for FALL-39 (Ageberth, G., Gunne, H., Odeberg, J., Kogner, P., Boman, H.G. & Gudmundsson, G.H. (1995) FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis, Proc. Natl. Acad. Sci. USA 92, 195-199).

Within the term "functional derivatives" used herein are included the peptides with free carboxyl groups and also acid

addition salts. Therefore the invention is not restricted to the specific peptides disclosed.

TABLE 1

Sequences of Rana temporaria skin peptides and relative amount in the secretion. Peptides for which the structure of the corresponding precursor has been predicted from cDNAs are marked with the asterisk. *a* indicates an amidated COOH-terminus. MLP, melittin-like peptide. Identical residues are boldfaced. Gaps (-) were inserted to maximize identities.

Peptide	Sequence	Yield
		nmol/mg
Temporin A	FLPLIGRVLSGILa	14.5
Temporin B*	LLPIVGNLLKSLLa	19.4
Temporin C	LLPILGNLLNGLLa	37.5
Temporin D	LLPIVGNLLNSLLa	1.1
Temporin E	VLPIIGNLLNSLLa	1.2
Temporin F	FLPLIGKVLSGILa	13.5
Temporin G*	FFPVIGRILNGILa	16.8
Temporin H*	LSP---NLLKSLLa	8.7
Temporin K	LLP---NLLKSLLa	9.8
Temporin L	FVQWFSKFLGRILa	3.6
MLP	FIGSALKVLAGVLPSVISWVK---Qa	5.1
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQa	

TABLE 2 Antimicrobial and lytic activity of Rana temporaria peptides. Lethal concentrations were calculated from inhibition zones on agarose plates seeded with the respective organisms. The data for cecropin are from Hultmark et al. (1983). S. Pyogenes β hem. group A and Ps.aeruginosa ATCC15692 are clinical isolates kindly provided by Dr. Paolo Visca, Institute of Microbiology, University of Rome La Sapienza. NT, not tested.

Organism and strain	Lethal concentration of					
	Temporin A	Temporin B	Esculentin 1	Cecropin A	MLP	Melittin
	μM					
B.megaterium BmII	1.2	2.8	0.1	0.5	NT	0.6
S.aureus Cowan1	2.3	6.0	0.4	>200	NT	0.2
Y.pseudotubercolosis	7.0	7.0	NT	0.5	NT	NT
S.pyrogenes βhem.group A	2.0	7.0	NT	NT	NT	NT
E.coli D21	11.9	21.0	0.2	0.3	NT	0.8
Ps. aeruginosa ATCC15692	>360	>360	0.7	NT	NT	NT
C.albicans	3.4	4.0	0.5	NT	NT	NT
Human red cells	>120	>120	>200	>400	0.5	0.9

TABLE 3. Antibacterial activity of temeporins A and B against E.coli D21 and related LPS modified strains. Assays were performed in LB broth/1% agarose, in the absence or in the presence of medium E (Vogel & Bonner 1956). Bacterial strains were kindly provided by Prof.H.G.Boman, University of Stockholm

Compound	Lethal concentration for				
	D21	D21e7	D21f1	D21f2	D22
	μM				
Temporin A	11.9	1.4	0.9	4.8	3.4
Temporin A + medium E	5.3	1.4	3.0	2.0	0.4
Temporin B	21.0	13.2	10.0	3.3	11.2
Temporin B + medium E	3.4	4.2	3.5	9.3	12.2

SEQUENCE LIST

Clone Rt-5

```
1   ACAATTCTGAGCCAACTGAACCACCCGAGCCCAAAGATGTTCACCTTGAAGAAATCCCTG
                                              M  F  T  L  K  K  S  L
61  TTACTCCTCTTTTTCCTTGGGACCATCAACTTATCTCTCTGTGAGGAAGAGAGAAATGCA
     L  L  L  F  F  L  G  T  I  N  L  S  L  C  E  E  E  R  N  A
121 GAAGAAGAAAGAAGAGATGAACCAGATGAAAGGGATGTTCAAGTGGAAAAACGACTTTTA
     E  E  E  R  R  D  E  P  D  E  R  D  V  Q  V  E  K  R  L  L
181 CCAATTGTTGGAAACCTGCTCAAGAGCTTGTTGGGAAAATAACCAAAAATGTTAAGAATG
     P  I  V  G  N  L  L  K  S  L  L  G  K  +
241 GAATTGGAAATCATCTGATGTGGAATATCATTTAGCTAAATGAGCAACAGATGTCTTATT

301 TAAAAAAATAAATATGTTCCATC
```

Clone Rt-6

```
1   GCTTTGTAGGATAGACCTGCACTGAAGTCTTCCAGCCGTCTACATTCTGAGCACCAACTG

61  AACTACCCGAGCCCAAAGATGTTCACCTTGAAGAAATCCCTGTTACTCCTCTTTTTCCTT
                          M  F  T  L  K  K  S  L  L  L  L  F  F  L
121 GGGACCATCAACTTATCTCTCTGTGAGGAAGAGAGAAATGCAGAAGAAGAAAGAAGAGAT
     G  T  I  N  L  S  L  C  E  E  E  R  N  A  E  E  E  R  R  D
181 GAACCAGATGAAAGGGATGTTCAAGTGGAAAAACGACTTTCACCAAACCTGCTCAAGAGC
     E  P  D  E  R  D  V  Q  V  E  K  R  L  S  P  N  L  L  K  S
241 TTGTTGGGAAAATAACCAAAAATGTTAAGAATGGAATTGGAAATCATCTGATGTGGAATA
     L  L  G  K  +
301 TCATTTAGCTAAATGCGCAACAGATGTCTTATTTAAAAAATAAATATGTTGCATAC
```

Clone Rt-17

```
1   CCCCTCCAGCTGTCTACATTCTCATAACCAACTGAACCACCCGAGCCCAAAGATGTTCAC
                                                      M  F  T
61  CTTGAAGAAATCCCTCTTACTCCTTTTCTTCCTTGGGACCATCAACTTATCTCTCTGTGA
      L  K  K  S  L  L  L  L  F  F  L  G  T  I  N  L  S  L  C  E
121 GGAAGAGAGAGATGCCGATGAAGAAAGAAGAGATGATCTCGAAGAAAGGGATGTTGAAGT
      E  E  R  D  A  D  E  E  R  R  D  D  L  E  E  R  D  V  E  V
181 GGAAAAGCGATTTTTTCCAGTGATTGGAAGGATACTCAATGGTATTTTGGGAAAATAACC
      E  K  R  F  F  P  V  I  G  R  I  L  N  G  I  L  G  K  +
241 AAAAAAAGTTAAAACTTTGGAAATGGAATTGGAAATCATCTAATGTGGAATGTCATTTAG

301 CTAAATGCACATCAAATGTCTTATAAAAA
```

CLAIMS

1. A polypeptide selected from the following peptides:

F L P L I G R V L S G I L - amide
L L P I V G N L L K S L L - amide
L L P I L G N L L N G L L - amide
L L P I V G N L L N S L L - amide
V L P I I G N L L N S L L - amide
F L P L I G K V L S G I L - amide
F F P V I G R I L N G I L - amide
L S P N L L K S L L - amide
L L P N L L K S L L - amide
F V Q W F S K F L G R I L - amide
G L L S G L K K V G K H V A K N V A V S L M D S L K C K I S G D C

and functional derivatives and pharmaceutically acceptable salts thereof.

2. One or more polypeptides according to claim 1 for therapeutic use.

3. One or more polypeptides according to claim 2 for antimicrobial use.

4. One or more polypeptides according to claim 4 for antibacterial or fungal use.

5. The use of one or more polypeptides according to any preceding claim for the manufacture of a medicament having antimicrobial activity.

6. The use according to claim 5, wherein said medicament is in possession of antibacterial or antifungal activity.

7. A pharmaceutical composition containing as an active ingredient one or more polypeptides according to claim 1 in an effective amount together with a pharmaceutically acceptable carrier or diluent.

8. A pharmaceutical composition according to claim 7, wherein said amount is antimicrobially active.

9. A pharmaceutical composition according to claim 8, wherein said amount is antibacterially or antifungally

active.

10. A pharmaceutical composition according to any one of claims 7 to 9, wherein said carrier or diluent is adapted for oral, intraveneous, intramuscular or subcutaneous administration.

11. A cDNA clone having the sequence selected from the sequences shown as clone Rt-5, Rt-6 and Rt-17.

12. A method for inhibiting microbial growth in animals, such as mammals including man, comprising the step of administering to an animal subject to a disorder caused by antimicrobial attack one or more polypeptides according to claim 1 or a composition according to any one of claims 7 to 10 in an inhibitory amount.

13. A method according to claim 12 for inhibiting bacterial or fungal growth.

14. A method according to claim 12 or 13, comprising administration by injection of a conposition according to any one of claims 7 to 10 in an injectable dose form.

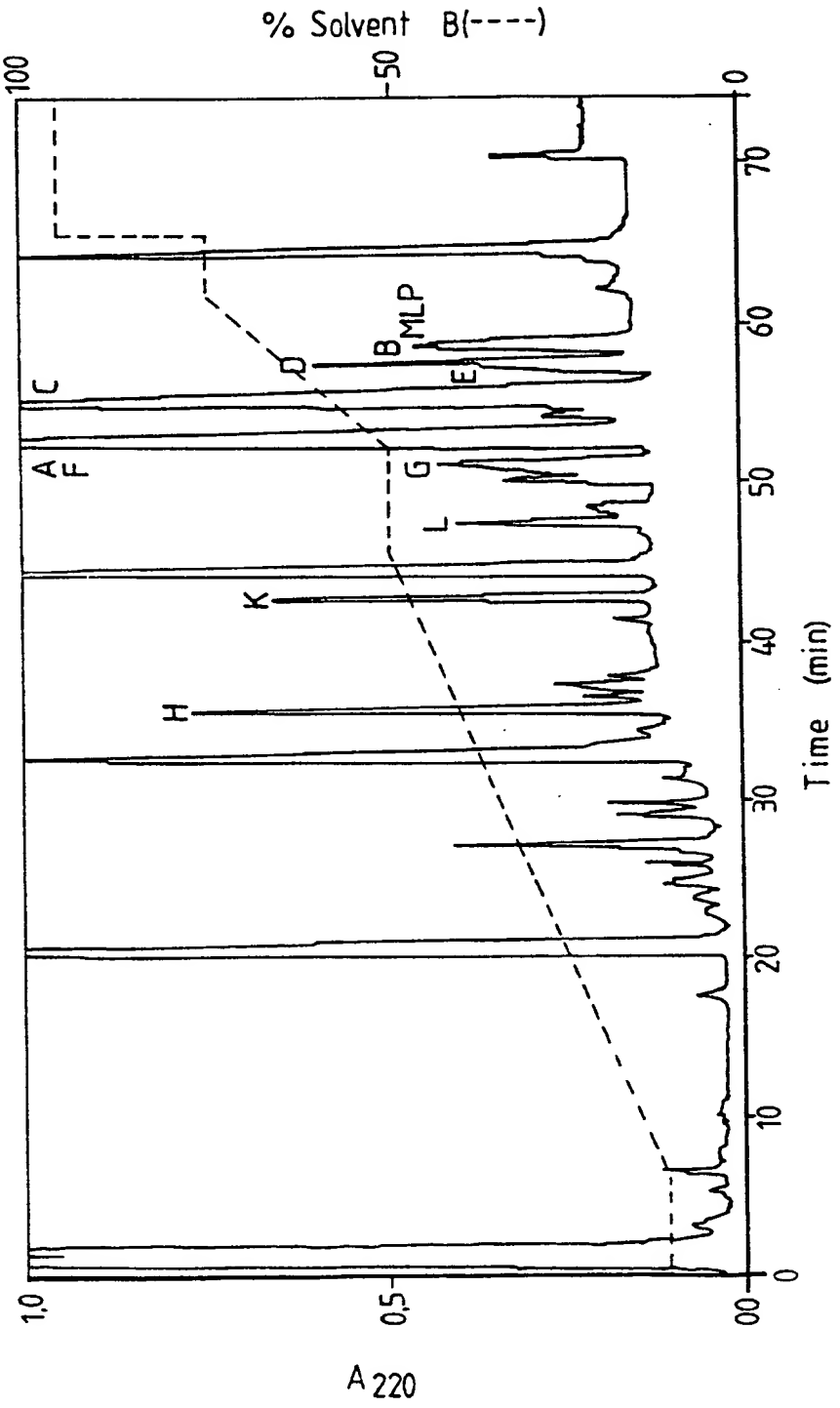

Fig. 1

Reverse-phase HPLC of skin secretion of R. *temporaria*. The elution position of the peptides reported in Table 1 is indicated by the corresponding letters. For details, see the Materials and Methods section.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/02075

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/46, C07K 7/08, A61K 35/56
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, REG, WPI, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Eur. J. Biochem., Volume 242, 1996, Maurizio Simmaco et al, "Temporins, antimicrobial peptides from the European red frog Rana temporaria" page 788 - page 792 --	1-11
A	TIBTECH, Volume 13, June 1995, Donatella Barra et al, "Amphibian skin: a promising resource for antimicrobial peptides" page 205 - page 209 --	1-11
A	WO 9527728 A1 (LUMINIS PTY. LIMITED), 19 October 1995 (19.10.95) --	1-11

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 April 1998	07 -04- 1998
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Gómez Lagerlöf Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/02075

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 12-14
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/03/98

International application No.
PCT/SE 97/02075

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9527728 A1	19/10/95	AU 2131995 A	30/10/95
		AU PM489194 D	00/00/00